MECHANOBIOLOGY OF MICE CERVIX: EXPRESSION PROFILE OF MECHANO-RELATED MOLECULES DURING PREGNANCY

by

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ABSTRACT
MECHANOBIOLOGY OF MICE CERVIX: EXPRESSION PROFILE OF MECHANO-RELATED MOLECULES DURING PREGNANCY (May 2018)

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There is a known reciprocation between the chronic exertion of force on tissue and both increased tissue density (*e.g.* bone) and hypertrophy (*e.g.* heart). This can also be seen in cervical tissue where the excessive gravitational forces associated with multiple fetal pregnancies promote preterm births. While there is a well-known regulation of cervical remodeling (CR) by sex steroid hormones and growth factors, the role of mechanical force is less appreciated. Using proteome-wide technology, we previously provided evidence for the presence of and alteration in mechano-related signaling molecules in the mouse cervix during pregnancy. Here we profile the expression of select cytoskeletal factors (Filamin A, gelsolin, vimentin, actinin-1, caveolin-1, transgelin, keratin-8, profilin-1) and their associated signaling molecules [Focal adhesion kinase (FAK) and the Rho-GTPases Cdc42, RhoA, and RhoB] in cervices of pregnant mice by real time PCR and confocal immunofluorescent microscopy. Messenger RNA and protein levels increased for each of these 12 factors, except for 3 (keratin-8, profilin-1, RhoA) that decreased during the course of pregnancy, and this corresponded with an increase in gravitational force exerted by the fetus on the cervix. We therefore conclude that size or weight of the growing fetus likely plays a key role in CR through mechano-transduction processes.

DEDICATION

This work is dedicated to my entire immediate family.
Notably my parents
for without their support and guidance, I would not be here.

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INTRODUCTION

When tissues, such as bone, skeletal muscle, and heart are exposed to chronic force they respond by either increasing their density (*e.g.* bone) or undergoing hypertrophy (*e.g.* heart) (Cowin & Hegedus 1976, Choukroun *et al*. 1999). Moreover, such changes, in reciprocal fashion, serve to further increase the mechanical forces in these tissues. We suspect a similar situation occurs in the cervix during multiple fetal pregnancies, where the developing fetuses exert an excessive gravitational force that nearly always results in preterm births (Nott *et al*. 2016, Myers *et al*. 2015). Cervical remodeling (CR) is a complex biological process involving multiple factors (Jorge *et al*. 2014, Mahendroo 2012, Holt *et al*. 2011, Timmons *et al*. 2010). Most of our previous studies of CR regulation have focused on chemical cues, such as sex steroid hormones and growth factors (Stanley *et al*. 2015, 2018, Zhao *et al*. 2013, Donnelly *et al*. 2013). However, more recently we used a proteome-wide technology to provide the first evidence of the presence and expression profile of mechano-related signaling molecules in the mouse cervix during pregnancy and cervical repair (Schwabe *et al*. 2014, Stanley *et al*. 2018).

CR can be divided into four unique overlapping stages: cervical softening, ripening, dilation, and postpartum repair (Read *et al*. 2007, Timmons *et al*. 2010, Jorge *et al*. 2014). In mice, the softening phase of CR occurs between day 10 and day 12 of a 19-day gestation period. Ripening occurs at day 18 (Read *et al*. 2007). In humans these processes begin in the first trimester (Timmons *et al*. 2010, Myers *et al*. 2010). Together, these biochemical and physical alterations transform the cervix from a sturdy and rigid structure to a dynamic and

flexible one, that both maintains the fetus *in utero* while readying the cervix for a timely birth (Timmons *et al*. 2010, Jorge *et al*. 2014, Mahendroo 2012, Holt *et al*. 2011). While preterm birth has long been associated with abnormal acceleration towards ripening and dilation, only recently have there been insights into its molecular mechanisms (Holt *et al*. 2011), including the role of endocrine factors, notably the progesterone and estrogen ratio (Mahendroo, 2012). As the influence of both endocrine and mechanical cues on neighboring reproductive tissue (*i.e.* uterus) has been clearly demonstrated, a similar effect is likely to be seen in the cervix as well (Shynlova *et al*. 2009).

The cervix appears to serve dual functions. Firstly, by remaining closed until full term, it bears the ever-increasing gravitational force exerted on it by the fetus. Secondly, the cervix must simultaneously begin to transform into a state that eventually will be conducive to dilation, thus allowing for timely fetal delivery at parturition (Jorge *et al*. 2014, Leppert 1995). Clearly, the collagen-rich ECM of the cervix must also undergo significant changes during CR (Read *et al*. 2007, Word *et al*. 2007, Leppert *et al*. 2014, Jorge *et al*. 2014, Orr *et al*. 2006, Zhang *et* al. 2012, Yoshida *et* al. 2014, Myers *et al*. 2010, Liu *et al*. 1997, Aspden 1988). This also is true of the uterus where there is a relationship between force and mechanotransduction in normal pregnancy and the pathogenesis of uterine fibroids (Leppert *et al*. 2014, Wu *et al*. 2008).

While extensive studies on cervical tissue ECM, morphology, and mechanical behavior at the micro and macro level have been undertaken, the mechanically-associated intracellular changes and underlying molecular mechanism of CR have not been studied. Our recent proteomic and microarray studies were the first to reveal evidence of the presence of

and alteration in mechano-related molecules, and the most dominantly expressed protein group were cytoskeletal molecules (Mowa *et al*. 2008, Schwabe *et al*. 2014).

This present study builds on these initial observations by characterizing the intracellular expression profile and cellular localization patterns of these cytoskeletal molecules (cross-linkers, assembly/dis-assembly) along with mechanosensors and other mechanosignaling molecules, as well as their potential role during CR in normal pregnancy and preterm labor.

MATERIALS AND METHODS

Animal Model

Pregnant C57BL6/129SvEv mice (*Mus musculus*) (days 11, 17, and 19) between 10-12 weeks old and non-pregnant mice sourced from Charles Rivers (Wilmington. MA) were used in these studies (n = 5), as described below. Ovariectomy was performed on all non-pregnant mice, as previously described (Donnelly *et al*. 2013), in order to eliminate confounding effects of endogenous ovarian hormones. Prior to tissue harvest, animals were administered with a lethal dose of euthasol (Euthasol®, Virbac animal health, Fort Worth, TX), perfused intra-cardially followed immediately by harvest of cervical tissues. Tissues were carefully harvested under a stereomicroscope to avoid any tissue contamination from uterine or vaginal tissues and then stored at -80^{o}C and or processed appropriately. All animals were housed at room temperature (RT), with a 12 h light: 12 h darkness cycle and animals had *ad libitum* access to water and feed. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the local institution (Appalachian State University) and the NIH guidelines (NIH publication number 86-23). Every effort was made to minimize animal suffering and numbers of animals used. Tissues were analyzed using molecular biology techniques (Confocal immunofluorescence and real-time PCR) in order to examine the specific mRNA and protein expression profile of select mechano-sensitive molecules and their associated signaling factors in cervical tissues during CR of mice.

Techniques

mRNA expression of select cytoskeletal and RhoGTPase proteins in mice cervix:

Gene expression of select cytoskeletal and RhoGTPase of mice cervix of pregnant and non-pregnant mice was examined using real-time PCR, as described previously using three main steps, namely **i**) RNA extraction, **ii**) cDNA generation and **iii**) Real time PCR (Donnelly *et al*. 2013, Mowa *et al.* 2008).

i) *Total RNA extraction*: Animals were euthanized and transcardially perfused with normal saline at the appropriate pregnancy time points described earlier. The cervices were harvested and immediately stored at -80°C. Total RNA was isolated from the cervices of individual animals using RNeasy Mini Kit (Qiagen, Valencia, CA). The amount and purity of total RNA for each sample was estimated by spectrophotometric analysis at A260 and A280 using Nanodrop (ThermoScientific, Waltham, MA). Aliquots of total RNA were diluted in RNase-free water and stored at -80°C.

ii) *cDNA generation*: Briefly, total RNA from mice cervices were reverse transcribed and amplified by reverse transcriptase enzyme (M-MLV) with reagents (Invitrogen, Carlsbad, CA) using an Eppendorf Master Cycler, according to the manufacturer's instructions, as described previously (Donnelley et al., 2013). No reverse transcriptase was added to negative control groups.

iii) *Real time PCR*: The generated cDNA was used to evaluate the comparative expression of 12 select genes (see Table 1) in cervices of non-pregnant and pregnant mice. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA), which contain pre-designed and pre-optimized gene-specific probe sets, were used to amplify specific target

genes, as previously described (Donnelly *et al*. 2013). cDNA amplification was performed using the Applied Biosystems 7500 Real-Time PCR system (Foster City, CA). The PCR reactions were set up in 96-well plate in volumes of 25 µL per well. The reaction components were as follow: 5 µL of cDNA, 12.5 µL of TaqMan Universal PCR Mastermix, 1.25 µL of Assays-on-demand™ gene mix (gene-specific), and 6.25 µL of RNase-free water. The program was set as follows: an initial step at 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 60 s. The comparative amount of mRNA was calculated from the threshold cycles with the instrument's software (7500 software v2.0, Applied Biosystems) according to the manufacturer's instructions. The relative expression levels of the target genes were normalized using an endogenous control gene, *Gusβ.* Non-template controls were used as controls for DNA contamination.

Protein expression and cellular localization of select cytoskeletal and RhoGTPase proteins in mice cervix using confocal immunofluorescence microscopy:

Confocal immunofluorescence microscopy was performed in order to subjectively determine changes in the expression of the target proteins, as well as to localize their cellular expression in the cervix. The proteins of interest that were investigated included mechano-sensitive signaling molecules (Cdc42, RhoA, RhoB) and cytoskeletal proteins [vimentin, phosphorylated caveolin-1 (Tyr 14), phosphorylated Focal Adhesion Kinase (Tyr 397)] and tissues were processed as described below. These studies complemented the quantitative expression of mRNA, by real-time PCR, described earlier.

i) *Tissue Processing*: Harvested tissues were immediately fixed in 10% formalin for 48 h, dehydrated and embedded in paraffin wax. The embedded tissues were later sectioned

at 5 μm, and the sections were processed for confocal immunofluorescence, as described previously (Donnelly *et al*., 2013, Stanley *et al*., 2015, 2018).

ii) *Confocal Immunofluorescence Microscopy*: Following deparaffinization and rehydration, tissue sections were initially incubated with either 10% normal goat serum (Cdc42, RhoA, RhoB, vimentin) or 10% normal mouse serum (p-caveolin-1, p-FAK) in 0.1 M PBS for 20 min at RT in order to block non-specific protein binding. Next, the sections were washed thrice in 0.1 M PBS and then incubated overnight at 4°C with primary antibodies [1:100 for p-FAK, 1:50 for the other 5 proteins of interest (Santa Cruz Biotech., Santa Cruz, CA)](see Table 2). The following day, the slides were washed thrice in 0.1 M PBS and were immediately incubated in diluted fluoro-tagged secondary antibody at 1:100 dilution (sc-2781, sc-2490, sc-2492, Santa Cruz Biotech., Santa Cruz, CA) for 45 min at RT. Following this, the slides were washed thrice with 0.1 M PBS and then counterstained with 5μM Sytox Green™ (ThermoFisher) according to the manufacture's guidelines. Slides were mounted with Ultracruz Mounting Medium (Santa Cruz Biotech., Santa Cruz, CA) and examined with a laser scanning confocal microscope (Carl Zeiss, Peabody, MA, USA).

Statistical analysis

Data for real-time PCR analysis were analyzed using ANOVA, followed by Holm-Sidak test for multiple comparisons. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

RhoGTPase in the mouse cervix are differentially expressed during gestation

As gestation advanced through day 19, the expression of Cdc42 and RhoB mRNAs and proteins increased in mice cervix relative to that of non-pregnant mice, as revealed by real time PCR and confocal immunofluorescence microscopy, respectively (Figs. 1 and 2). Specifically, the mRNA levels of Cdc42 increased by 3.7-fold at day 11 and 3.6-fold for RhoB at day 17, respectively (Figs. 1 and 2). Both proteins were largely localized in the cervical epithelial cells, with some sparse expression in stromal cells during late pregnancy (day 19). This was in marked contrast to the expression of RhoA mRNA and protein, which decreased drastically with advancing gestation relative to non-pregnant mice (Fig. 3). Indeed, by day 17 of pregnancy, the magnitude of RhoA mRNA expression was reduced to less than 5% of control.

Levels of vimentin, FAK and caveolin-1 in the mouse cervix increase during gestation

We next focused on a series of molecules important for cytoskeletal regulation, including vimentin, FAK, and caveolin-1. Vimentin is an intermediate filament protein common to mesenchymal cells or in epithelial cells following the epithelial to mesenchymal transition (EMT); FAK is an important signaling protein for cell adhesion and migration; and caveolin-1 classically aids in receptor-mediated endocytosis, but like FAK can be involved in

integrin signaling during adhesion and migration. The mRNA expression of vimentin, FAK and caveolin-1 increased in mice cervix over the course of pregnancy by 2.2-fold for vimentin and FAK and a remarkable 14-fold for caveolin-1 by day 17 relative to non-pregnant mice (Figs. 4, 5 and 6). The expression pattern for protein of the three molecules (vimentin, p-FAK, and p-caveolin-1) was similar to the pattern of their mRNA expression, i.e., they increased with gestation. Moreover, these proteins were largely localized in the epithelial cells of the remodeling cervix (Figs. 4, 5, and 6).

Actin accessory factors in mice cervix are differentially expressed during gestation

The expression pattern of actin accessory molecules, including filamin-1, gelsolin, actinin-1, transgelin, profilin-1 and keratin-8 were found to be differentially expressed during gestation (Figs. 7 and 8). Specifically, the mRNA expression of most of the molecules increased relative to non-pregnant mice, including filamin-1, gelsolin, actinin-1 and transgelin (Fig. 7). The specific peaks in mRNA expression at various time points were as follow: 7.1-fold for filamin-1 at day 11; 6.2-fold change for gelsolin at day 17; 2.7-fold change for actinin-1 at day 17 and 3.1-fold change for transgelin at day 11. In contrast, both profilin-1 and keratin-8 showed a drastic decrease in expression in the cervix during gestation (Fig. 8). Like RhoA, the mRNA levels of these molecules also decreased to less than 5% of control group (Fig. 8).

DISCUSSION

Cells are influenced by various stimuli in their microenvironment, including biochemical, structural and mechanical signals (Geiger *et al*. 2001, Dufty *et al*. 2002). Although we have known the potential influence of fetal weight on CR, most of the work thus far has focused on sex steroid hormones and growth factors. The little we do know on mechanical stimulation is largely focused on tissue response to force at the macroscale and the composition and function of ECM factors (Myers *et al.* 2008, Yoshida *et al.* 2014, 2016). Here, we first examined the presence and cellular localization, chronological gestational expression pattern, and activation of intracellular mechanical machinery in the remodeling cervix of mice. Interestingly, most of the molecules in this study were largely localized to the cervical epithelium, with sparse expression in the stroma, suggesting a significant role for epithelia in mechanical signaling. It is important to note that cervical epithelial cells are known to play a key regulatory role in CR and are located at the interface between the external environment and the body and are thus well-positioned for such a key role. Specifically, some of its (cervical epithelial) roles include, providing mechanical and or physical mucus and immune barriers, fluid balance, clearance of particulates, cervical proliferation and repair following vaginal birth. Of relevance to the present study, during pregnancy, cervical epithelial cells are also exposed to increasing mechanical force loads exerted by the growing fetus. This mechanical force likely has profound regulatory effects on the function of epithelial cells and CR. This speculation is supported by our recent proteomic findings revealing that mechano-related proteins are the most abundantly expressed of all proteins in the mouse cervix. This is particularly notable as fetal-induced forces exerted on

the cervix increase during pregnancy and their (mechanical-related molecules) levels revert back after births, following removal of fetal force (Schwabe *et al*. 2014, Stanley *et al*. 2018). The present findings are consistent with our earlier data and further present key evidence that increasing fetal-induced force on the cervix during pregnancy likely up-regulates the expression of cytoskeletal molecules via FAK and Rho-type GTPases.

We investigated the expression pattern of select cytoskeletal-related molecules involved in the modification, assembly/disassembly (*Gsn, Tgln, Pfn1*), and binding (*Actn1, Fln1*) of actin during CR. Of these molecules, 4 (*Actn1, Gsn, Tgln, Fln1*) increased in expression at the mRNA level, while 1 (*Pfn1*) decreased. This increase in actin levels likely reflects elevated levels of actin assembly which commonly occurs in tissues undergoing remodeling and provides the driving force for changes in cellular shape during proliferation, events that are both likely observed during CR (reviewed by Shekhar *et al*. 2016). One of the major factors that alter actin organization is the milieu of cues the cell is exposed to in its microenvironment, such as ECM stiffness or compliance, *i.e.*, mechanical force. For instance, when vascular tissues are stretched, the contractile phenotype of their smooth muscle cells stabilizes in a process involving the actin cytoskeleton (Albinsoon *et al*. 2004). Filamin-1 also plays a role in mechanosensing and transmitting force, suggesting that the role of Filamin-1 is more than just enforcing stress fibers (Nakamura *et al.* 2015) (reviewed by Zeidan *et al*. 2003). Could the remodeling cervix constantly bombarded by the ever-increasing weight load of the fetus also stabilize in a similar way? Does the cervix continuously monitor changes in ECM stiffness and force transmission, and thus, in part, adjust and calibrate its responsiveness to the force and thereby hold the fetus *in utero*? The rise in levels of *Actn1* and *Fln1* and the subsequent bundling and branching of actin into

much stronger stress fibers that follow cross-linking appear to suggest so. These resultant structures (stress fibers) effectively transmit force from contractile machinery to ECM structures via focal adhesion (FA) proteins, which in turn play a critical role in inducing mechanically-activated signaling pathways (Paszek *et al*. 2005, Provenzano *et al*. 2009, Wozniak *et al*. 2003). However, mechanistic and functional studies are needed to evaluate these speculations.

Levels of *Gsn* and *Tgln* mRNA, which code for actin-modifying molecules, also increase, thus likely supporting actin polymerization over the course of pregnancy (Sun *et al*. 1999, Shekhar *et al.* 2016). In contrast, there is a decrease in *Pfn1* mRNA levels. This is consistent with previous findings that show that although profilin-1 is an essential driver of membrane protrusion during cell migration, high concentrations of profilin-1 do inhibit cell migration and lead to disappearance of filaments in lamellipodia (reviewed by both Shekhar *et al.* 2016, Ding *et al.* 2012). It is likely that cell motility events, such as lamellipodia formation and migration are not as critical during CR as maybe the case with proliferation, except perhaps for immune cell infiltration near term, which are believed to play a role in cervical repair.

We also observed an intriguing characteristic expression pattern by two other cytoskeletal molecules, vimentin and keratin, which form intermediate filaments (IFs). IF networks also play a role in mechanotransduction (Goldman *et al.* 1996, and reviewed by Qin *et al.* 2009). While both mRNA and protein levels of vimentin increased as expected, to our surprise mRNA levels of keratin-8, which ensures integrity of epithelial tissue, decreased in late pregnancy. Previous studies have shown that the loss of keratin-8/18 in simple epithelia decreases the local stiffness of FA (reviewed by Loschke *et al*. 2015). These unique

differences in the expression patterns of IFs and actin in the remodeling cervix is intriguing and future studies should investigate their interaction with ECM and their impact on CR.

Cellular mechanotransduction machinery can be divided into (i) a mechanosensing module, which contains molecular clutch proteins (*e.g.* talin, vinculin) that directly link integrin receptors with the actin cytoskeleton, (ii) mechanosignaling proteins (*e.g.* FAK, paxillin), (iii) cross-linking proteins (*e.g.* α-actinin, filamin), and (iv) actin polymerizing factors (*e.g.* gelsolin) (Stutchbury et al. 2017). FA complexes are located at cell-ECM attachment sites (Burridge *et al*. 1988). Different combinations of cytoskeletal molecules can assemble there (FA), depending on whether they are recruited by mechanical or chemical (growth factor) stimuli (Burridge *et al*. 1988). These molecules (Cytoskeleton), in turn, activate specific signaling pathways and control specific biological processes. FAK is a prominent member of the FA complex, is activated early during FA signaling and is critical for mechanosensing (Schaller *et al*. 1994, Schlaepfer *et al*. 1998). Since phosphorylation of FAK at tyrosine 397 by Src is associated with Stat 3 involved in growth regulation in other tissues (Schaller *et al*. 1994, Schlaepfer *et al*. 1998), the observed increase in levels of phosphorylated FAK at tyrosine 397 in the current study indicates the possible presence and activation of FAK-induced cervical growth. However, the exact biological process mediated by the activated FAK and the specific associated downstream signaling molecules during CR are currently unclear. An increase in Rho-mediated cell contraction in response to stiff microenvironments also drives FAK activation in FA (Pirone *et al*. 2006). Since FAK is stimulated by various factors, including mechanical ("outside-in" and "inside-out" forces) and chemical (VEGF, EGF, IGF) cues, it is unclear for now which of the two cues are responsible or whether they have a synergistic effect on FAK expression during CR. It is also

conceivable that the growing fetus may directly stimulate mechanical-sensitive ion channels which are present in the cervix, called piezol, to convert mechanical cues into biochemical signals (Lewis *et al*. 2017). This may be independent of the collagen-rich ECM whose stiffness in early pregnancy is likely to be a critical microenvironmental stimulus in mechanosensing, decreases as pregnancy advances. It is interesting to note that these channels and the cytoskeleton exhibit reciprocal regulation (Lewis *et al*. 2017). Alternatively, since mechano-sensitive cells are known to have mechanical memory, i.e. in stem cells, the magnitude of response to mechanical cues does not change over time even after the initial degree of stiffness is later altered, as is the case during CR, mechanical memory could play a role (Yang *et al*. 2014). Lastly, VEGF receptors, members of the receptor tyrosine kinase group, are abundantly expressed in the cervix where they help induce cervical growth, among other things (Donnelly *et al*. 2013) and are also known in other tissues to induce expression of FA molecules (Avraham *et al*. 2003). Perhaps FA proteins in the cervix cross talk with VEGF receptors during pregnancy. Future studies need to verify these speculations.

We also observed an increase in the expression of caveolin-1 mRNA and protein. Caveolae indentions in the cell membrane of aortic endothelial cells increase in number under laminar or physiological stress and when the tissue is stretched (Yu *et al*. 2006, reviewed by Shihata *et al*. 2016). Caveolae and caveolin-1 are also known to regulate cell cycle and senescence (Quest *et al*. 2002). Caveolin-1 is in addition believed to interact with other intracellular signaling molecules contained in caveolae. This includes G-coupled receptors and tyrosine kinases, and these interactions in turn stimulate downstream signaling, notably the members of the Rho GTPase family (Bender *et al*. 2002).

The latter half of pregnancy in mice is characterized by pronounced proliferation of the cervical tissue, largely dominated by epithelial cells (Burger & Sherwood 1998). The Rho-family of GTPases play a key upstream role in physiological and pathological processes of cellular transformation and proliferation using the mechanically-sensitive cytoskeleton (Wennerberg & Der 2004, Hall 1998, Karnoub & Der 2013, Fujiwara *et al.* 2016, Thorne *et al*. 2015). In the present study levels of Cdc42 and RhoB increased in the remodeling cervix, but not RhoA, which was down-regulated. Each of these molecules belongs to the Ras superfamily of small GTP-binding proteins (Downward 1990). Rho-type GTPases are known to regulate numerous actin-mediated signaling pathways (Paszek *et al*. 2005, Provenzano *et al*. 2009). Notably, they play a key regulatory role in the response by cells to ECM stiffness and respond to mechanical cues by inducing intracellular contractility via myosin motor proteins (Paszek *et al*. 2005, Provenzano *et al*. 2009), i.e., when motor proteins sense increased stiffness in the ECM, Rho is activated. This, in turn, leads to further induction of intracellular contractility with the goal of generating force that matches ECM stiffness, a mechanical equilibrium called tensional homeostasis (TH) (Wozniak *et al*. 2003). The magnitude and tone of TH determines the type of mechano-sensitive signaling pathways activated in FAs and consequently the biological event. In late CR, as collagen is remodeled and the ECM become more compliant, cervical stiffness diminishes, as does TH. Thus, increasing gravitational force exerted on the remodeling cervix by the weight of the growing fetus, and not ECM stiffness, maybe directly responsible for inducing the mechanical stimuli and the observed molecular readout in the present study. Also, like FAK, Rho is involved in proliferation (Provenzano *et al*. 2009). Notably, Rho is involved in cell-cycle progression at several stages and is also associated with various signaling pathways involved in

proliferation, including ERK1/2, p38 MAPK and JNK, serum response factor, and phosphoinositide 3 kinase-mediated activation of the Akt pathway (Sun *et al*. 2007, Provenzano *et al*. 2009). This pattern of protein expression observed in the present study along with the prominence of proliferation during CR collectively suggest a role for mechanotransduction signaling in CR.

The timing in the increase of Cdc42 expression in the cervical tissue observed here coincides with the proliferation of cells. Cdc42 is normally associated with cytoskeletal reorganization, filopodia formation, and motility (Wennerberg & Der 2004). Filopodia are used to probe the extracellular microenvironment. During fetal growth and CR, the ECM becomes very dynamic. The pattern of RhoA and RhoB during CR were opposite, with RhoA decreasing and RhoB increasing as pregnancy advanced. These Rho GTPases, which have as much as 85% homology (Wennerberg & Der 2004), have very similar downstream signaling molecules that mediate the re-organization of cytoskeletal structure and function, notably through the Rho-ROCK pathway (Paszek *et al*. 2005, Provenzano *et al*. 2009). To our surprise, the expression patterns of these two Rho GTPases in the remodeling cervix were different (decreased RhoA, increased RhoB). This difference in expression pattern is currently unclear. It is, however, important to note that traditionally RhoA contributes to structural integrity of the cell (Wennerberg & Der 2004). Further, a study by Fujiwara and others demonstrated a force-activated RhoA pathway in cultured cells that directly influences actin stress fibers and up- regulates keratin networks (specifically keratin 8/18) (Fujiwara *et al*. 2016). Here, similar to RhoA expression patterns, we note a decrease in keratin-8 mRNA expression as CR progressed. Our current findings regarding the expression trends of both RhoA and keratin correspond to that of Fujiwara and others (2016). Further, Thorne and

others also demonstrated a forced-induced RhoA/ROCK/MAPK/ERK proliferation pathway in myometrial tissue through actin and myosin-induced contractility (Thorne *et al*. 2015). The difference in our current findings and those observed earlier in myometrial tissue may be based on tissue-specific expression profiles. In the absence of RhoA's influence in the cervix, Cdc42 (discussed above) maybe the primary modulator of actin organization and mechanical-induced proliferation during CR.

In conclusion, the present study demonstrates the existence of dynamic expression patterns in cytoskeletal-related molecules in the remodeling cervix of mice during pregnancy. The prevalence of these cytoskeletal-related molecules, FA proteins, and Rho-type GTPases were largely localized in the epithelium, suggesting an important role for epithelial cells in mechanosignaling pathways. Our data provides a snapshot of dynamic cytoskeletal activity during CR and points to the importance of developing and further studying CR-specific signaling pathways that are mechanically stimulated (see proposed model, Fig 9). Future studies should attempt to delineate the specific roles of the different regulatory cues of CR and whether they synergize with mechanical stimulus. Also, there is need to examine whether an optimal clinically-relevant threshold ratio exists between fetal and cervical sizes during pregnancy.

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TABLES

Table 1

Select cytoskeletal (9) and RhoGTPase (3) proteins investigated in this study with their cell function. 75% of the targets analyzed exhibited an up regulated expression trend, while 25% of the targets exhibited a down regulated expression trend.

Protein name	Known function	Trend of protein and/or mRNA expression in pregnancy
Cdc42	GTPase regulating signaling in cell morphology & division	Up regulated
RhoA	GTPase associated with cytoskeletal organization	Down regulated
RhoB	GTPase associated with cytoskeletal organization	Up regulated
Vimentin (Vim)	Supporting and anchoring of organelles in the cytosol	Up regulated
p-Focal Adhesion Kinase (P-FAK)	Cellular adhesion and spreading processes	Up regulated
p-Caveolin-1 (P-Cav1)	Links integrin subunits to the tyrosine kinase FYN	Up regulated
Filamin-1 (Fln1)	Structural crosslink of actin filaments, aids in migration	Up regulated
Actinin-1 (Actn1)	Actin binding protein in microfilament bundles	Up regulated
Transgelin (Tagln)	Shape-change sensitive actin cross-linking/ gelling protein	Up regulated
Gelsolin (Gsn)	Regulator of actin filament assembly	Up regulated
Keratin-8 (Krt8)	Cellular structural integrity and signal transduction	Down regulated
Profilin-1 (Pfn1)	Actin polymerization regulation	Down regulated

Table 2

Select cytoskeletal (3) and RhoGTPase (3) proteins investigated by laser scanning confocal microscopy in this study were targeted using primary antibodies purchased from Santa Cruz Biotechnology, Inc.

Target protein name	Primary antibody source	Product number
Cdc42	Mouse monoclonal	sc-8401
RhoA	Mouse monoclonal	sc-418
RhoB	Mouse monoclonal	sc-8048
Vimentin (Vim)	Mouse monoclonal	sc-32322
p-Focal Adhesion Kinase (p-FAK) (Tyr 397)	Rabbit polyclonal	sc-11765-R
p-Caveolin-1 (p-Cav1) (Tyr 14)	Goat polyclonal	sc-14037

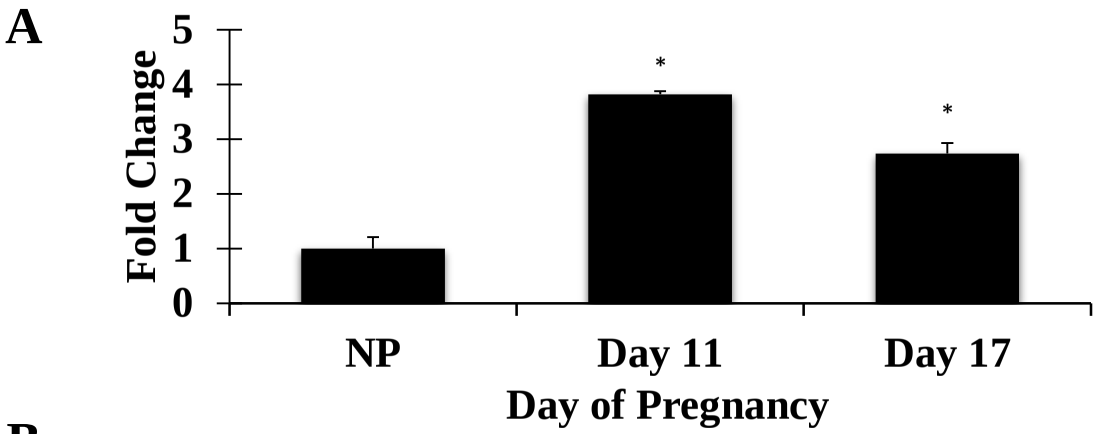

Figure 1

Overall Cdc42 mRNA and protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**). Real time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of Cdc42 mRNA in cervix tissue increases by day 11 of pregnancy as well as day 17 of pregnancy, though at lower intensity. (n=5; *P<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of Cdc42 protein expression was stronger at day 19 (b, c) compared to NP (a). The epithelial cell lining of the cervix tissue (notated as "E") was where high Cdc42 expression was localized in day 19 of pregnancy. Boxed-in area on image b shows epithelial expression localization as well as magnification of the area in image c. Tissue was incubated with Cdc42 and Texas red antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Any overlap appears as *yellow*, with strong Cdc42 appearing *red*. Magnification: a = X10, b = X10, c = X20. *L* lumen. *S* stromal cells. n=1.

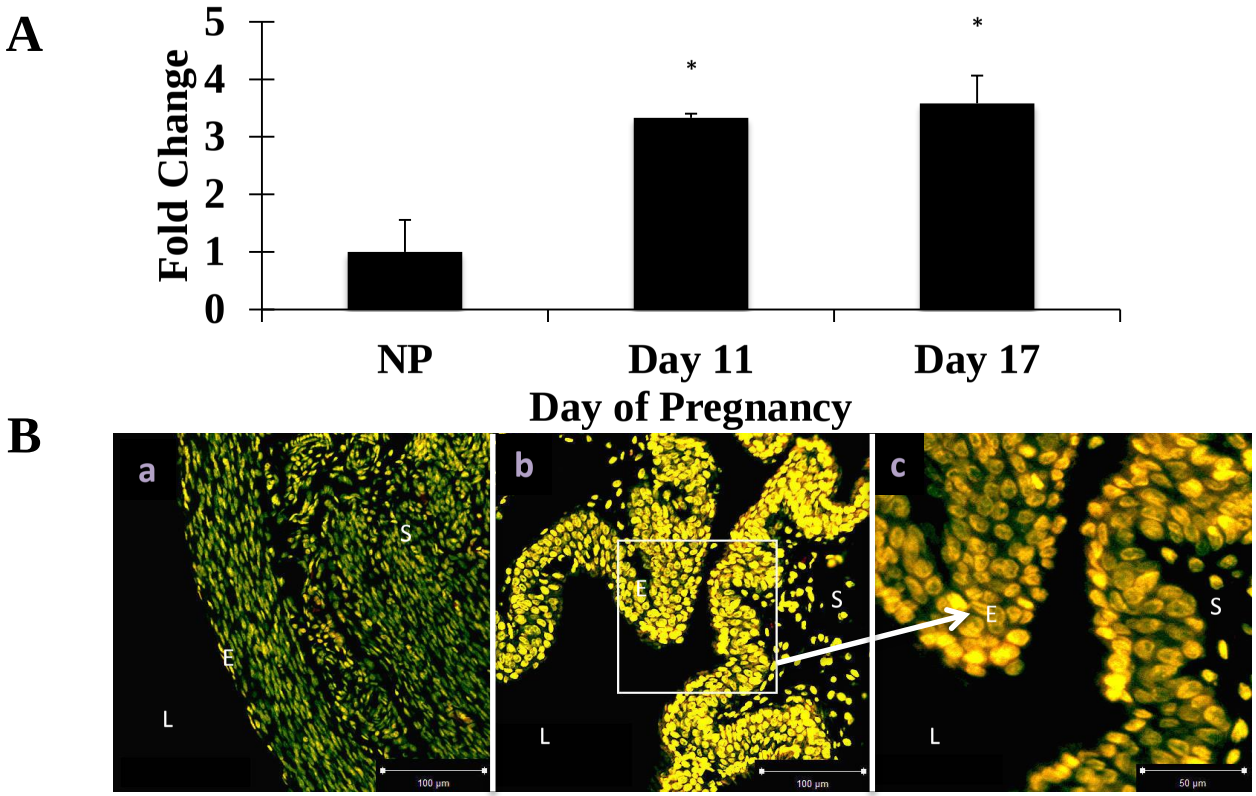

Figure 2

Overall RhoB mRNA and protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**). Real time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of RhoB mRNA in cervix tissue increases by day 11 of pregnancy as well as day 17 of pregnancy over 3 fold. (*n*=5, **P*<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of RhoB protein expression was stronger at day 19 (b, c) compared to NP (a). The epithelial cell lining of the cervix tissue (notated as "E") was where higher RhoB expression was localized in day 19 of pregnancy. Boxed-in area on image b shows epithelial expression localization as well as magnification of the area in image c. Tissue was incubated with RhoB and Texas red antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Overlap appears as *yellow*, with RhoB signal apparent as bright *yellow/red* combination. Magnification: a = X10, b = X10, c = X20. *L* lumen. *S* stromal cells. *n*=1.

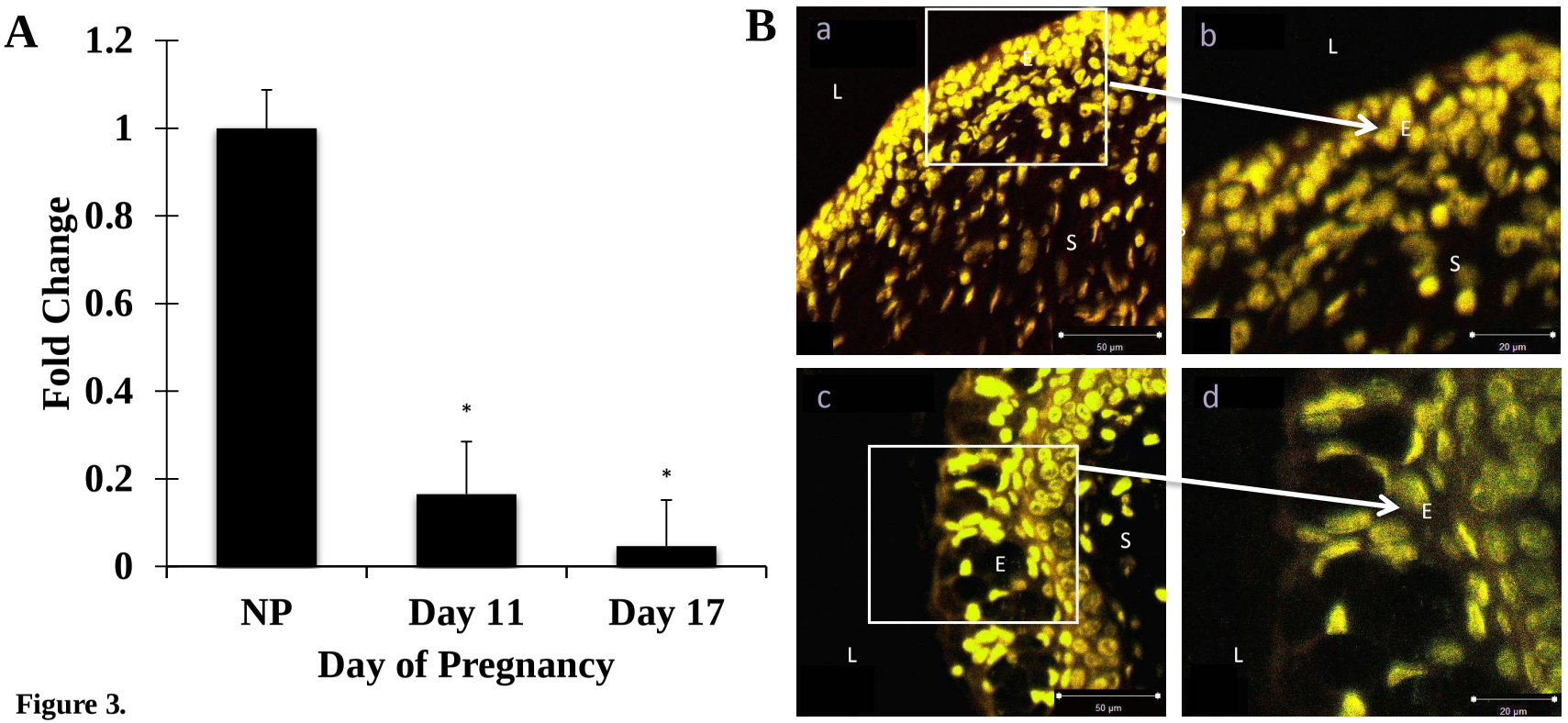

Figure 3.

Overall RhoA mRNA and protein expression sharply decreases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**)**.** Real time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of RhoA mRNA in cervix tissue decreases as much as 5 fold by day 11 of pregnancy, and further at day 17 of pregnancy. (n=5, *P<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of RhoA protein expression was slightly weaker at day 19 (c, d) compared to NP (a, b). The epithelial cell lining of the cervix tissue (notated as "E") was where noticeable RhoA expression differences was localized in both NP and day 19 of pregnancy. Boxed-in area on images a and c shows epithelial expression localization as well as magnification of the area in images b and d. Tissue was incubated with RhoA and Texas red antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Overlap appears as *yellow*, with RhoA signal apparent as bright *yellow/red* combination. Magnification: a = X20, b = X40, c = X20, d=X40. *L* lumen. *S* stromal cells. n=1.

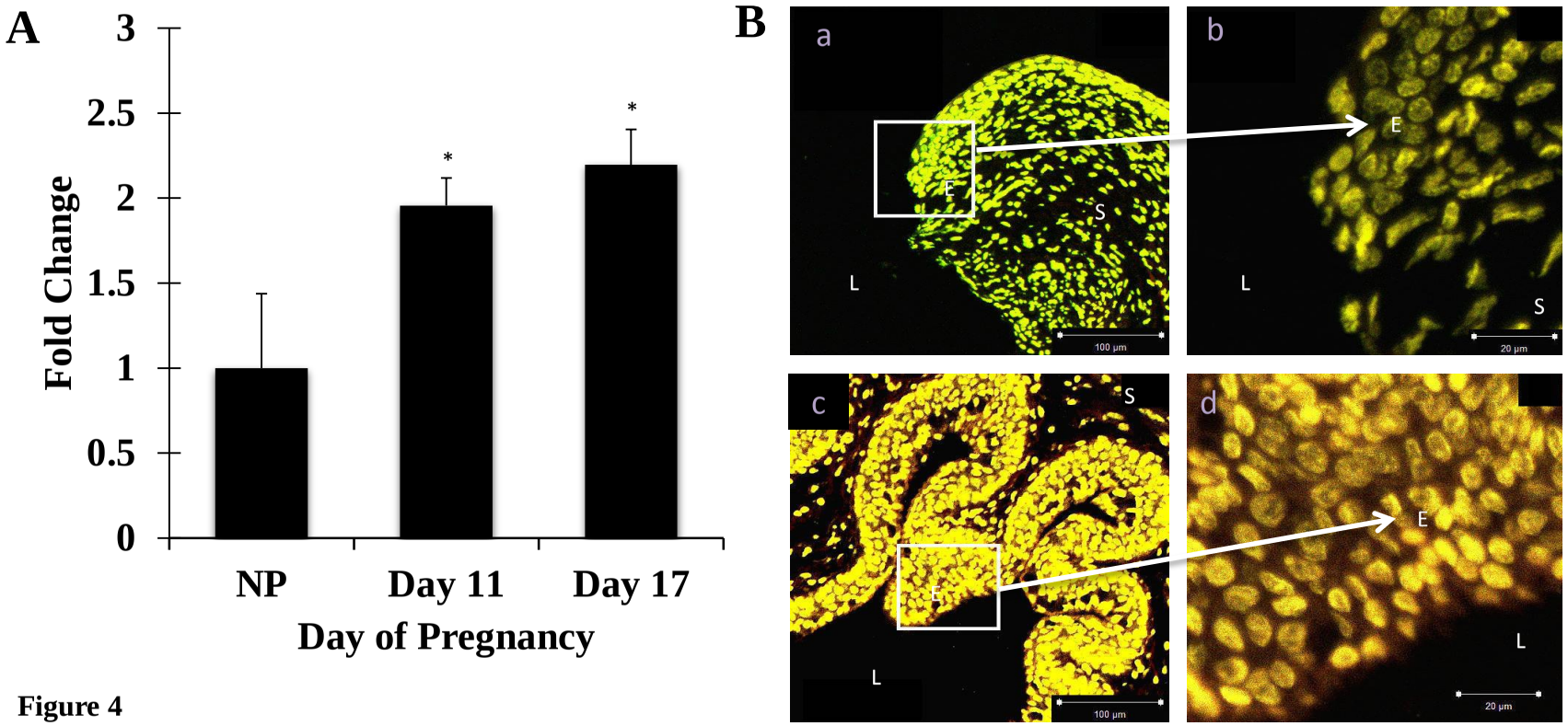

Figure 4

Overall Vimentin mRNA and protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**). Real-time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of Vimentin mRNA in cervix tissue increases by day 11 of pregnancy, and further at day 17 of pregnancy. (n=5, *P<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of Vimentin protein expression was stronger at day 19 (c, d) compared to NP (a, b). The epithelial cell lining of the cervix tissue (notated as "E") was where noticeable Vimentin expression differences was localized in both NP and day 19 of pregnancy. Boxed-in area on images a and c shows epithelial expression localization as well as magnification of the area in images b and d. Tissue was incubated with Vimentin and Texas red antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Overlap appears as *yellow*, with Vimentin signal apparent as bright *yellow/red* combination. Magnification: a = X10, b = X40, c = X10, d=X40. *L* lumen. *S* stromal cells. n=1.

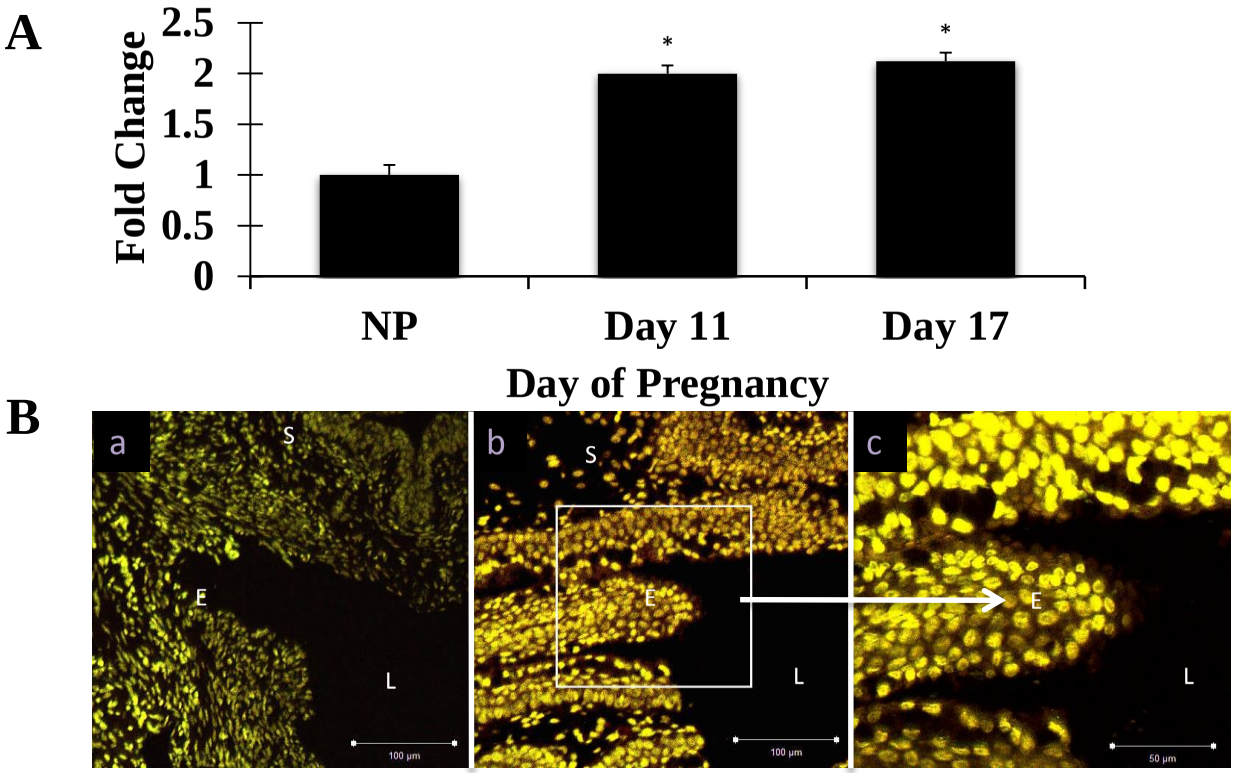

Figure 5

Overall Focal Adhesion Kinase (FAK) mRNA and p-FAK protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**). Real time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of FAK mRNA in cervix tissue increases by day 11 of pregnancy as well as day 17 of pregnancy. (n=5, *P<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of pFAK protein expression was stronger at day 19 (b, c) compared to NP (a). The epithelial cell lining of the cervix tissue (notated as "E") was where higher pFAK expression was localized in day 19 of pregnancy. Boxed-in area on image b shows epithelial expression localization as well as magnification of the area in image c. Tissue was incubated with pFAK (Tyr 397) and rhodamine antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Overlap appears as *yellow*, with pFAK (Tyr 397) signal apparent as bright *yellow/red* combination. Magnification: a = X10, b = X10, c = X20. *L* lumen. *S* stromal cells. n=1.

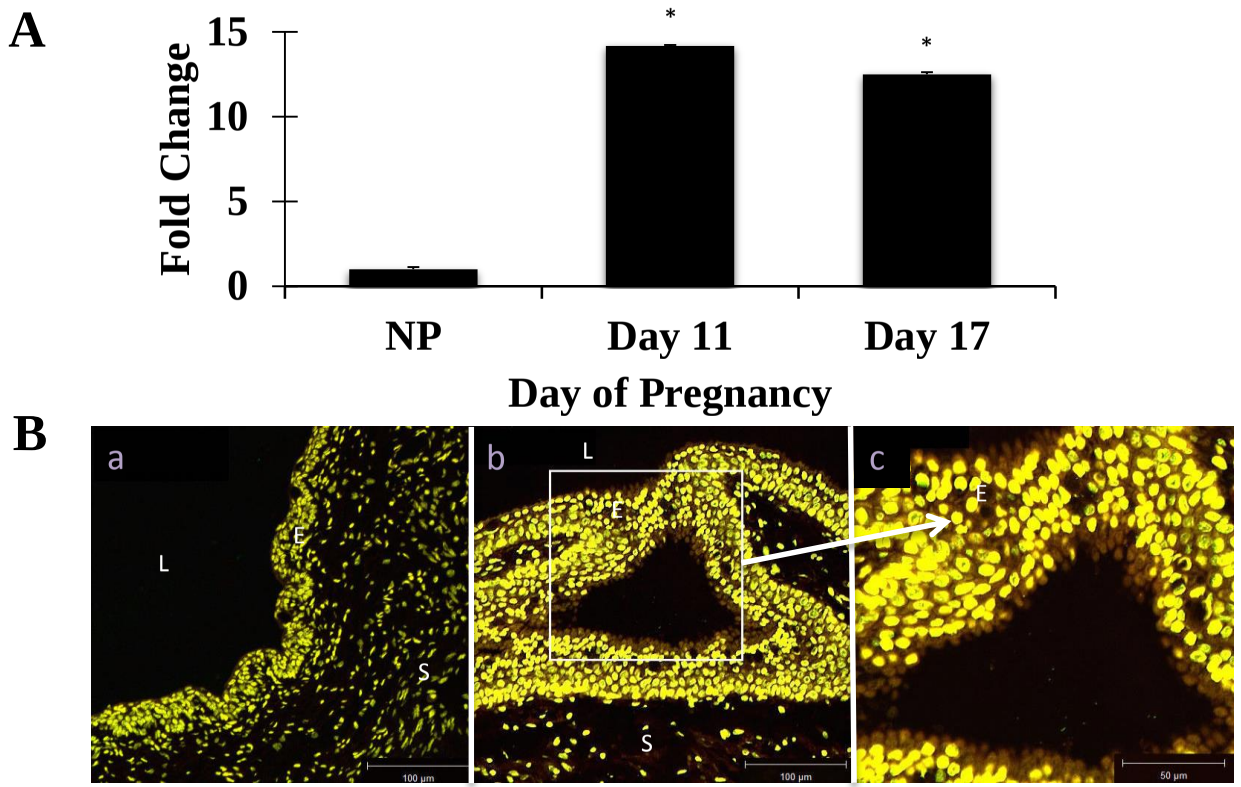

Figure 6

Overall Caveolin-1 (Cav-1) mRNA and pCav-1 protein expression increases over the course of pregnancy in mice cervix compared to non-pregnancy, as revealed by real time PCR (**A**) and confocal immunofluorescence (**B**). Real time PCR exhibited that in comparison to ovariectomized non-pregnant (NP) mice, expression of pCav-1 mRNA in cervix tissue increases by day 11 of pregnancy as well as day 17 of pregnancy. (n=5, *P<0.05 for days 11/17 vs. NP). Confocal immunofluorescence revealed that the intensity of pCav-1 protein expression was stronger at day 19 (b, c) compared to NP (a). The epithelial cell lining of the cervix tissue (notated as "E") was where higher pCav-1 expression was localized in day 19 of pregnancy. Boxed-in area on image b shows epithelial expression localization as well as magnification of the area in image c. Tissue was incubated with pCav-1 (Tyr 14) and rhodamine antibodies, and a nuclei-specific stain, i.e., Sytox Green™, staining *green*. Overlap appears as *yellow*, with pCav-1 (Tyr 14) signal apparent as bright *yellow/red* combination. Magnification: a = X10, b = X10, c = X20. *L* lumen. *S* stromal cells. n=1.

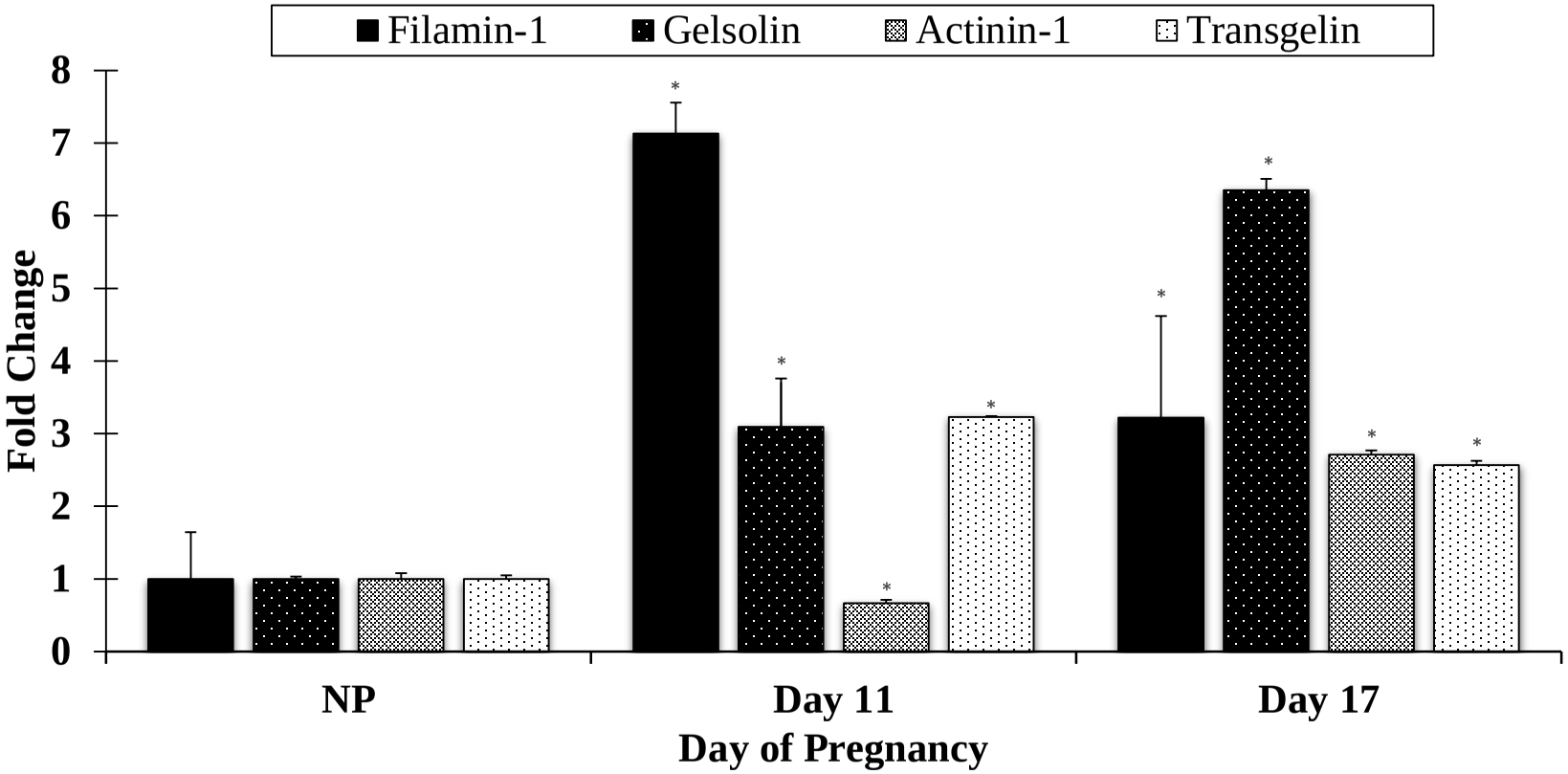

Figure 7
The effects of advancing pregnancy on mRNA expression of cytoskeletal proteins Filamin-1, Gelsolin, Actinin-1, and Transgelin in pregnant mice cervix tissue (days 11 and 17), as revealed by real time PCR. Overall, advancing pregnancy causes an increased expression for these four select cytoskeletal molecules, with all having increased by late pregnancy (day 17) compared to NP control. *n*=5; **P*=<0.05.

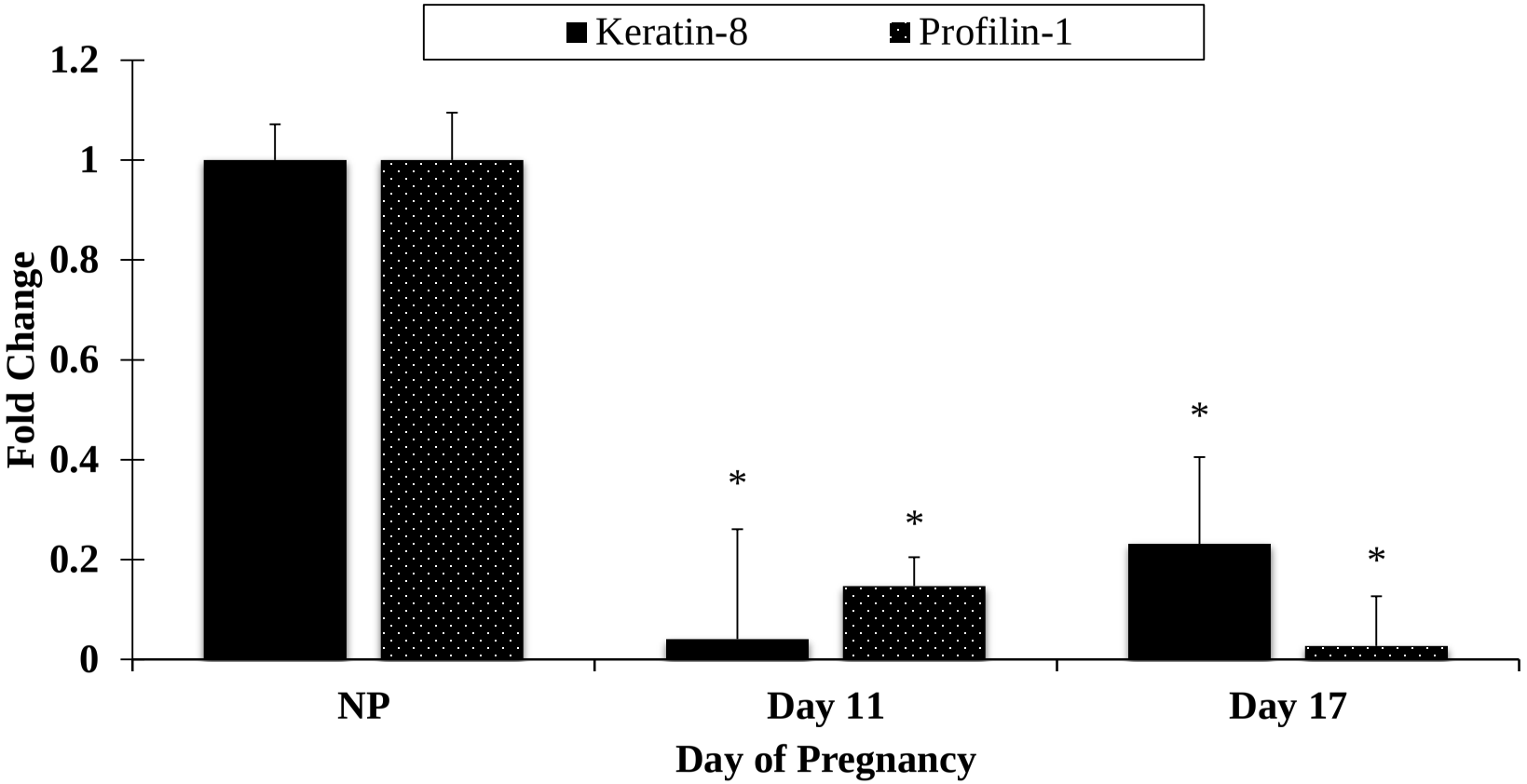

Figure 8
The effects of advancing pregnancy on mRNA expression of cytoskeletal proteins Keratin-8 and Profilin-1 in pregnant mice cervix tissue (days 11 and 17), as revealed by real time PCR. Overall, advancing pregnancy causes an decreased expression for these two select cytoskeletal molecules, with both having decreased by mid (day 11) and late (day 17) pregnancy compared to NP control. *n*=5; **P*=<0.05.

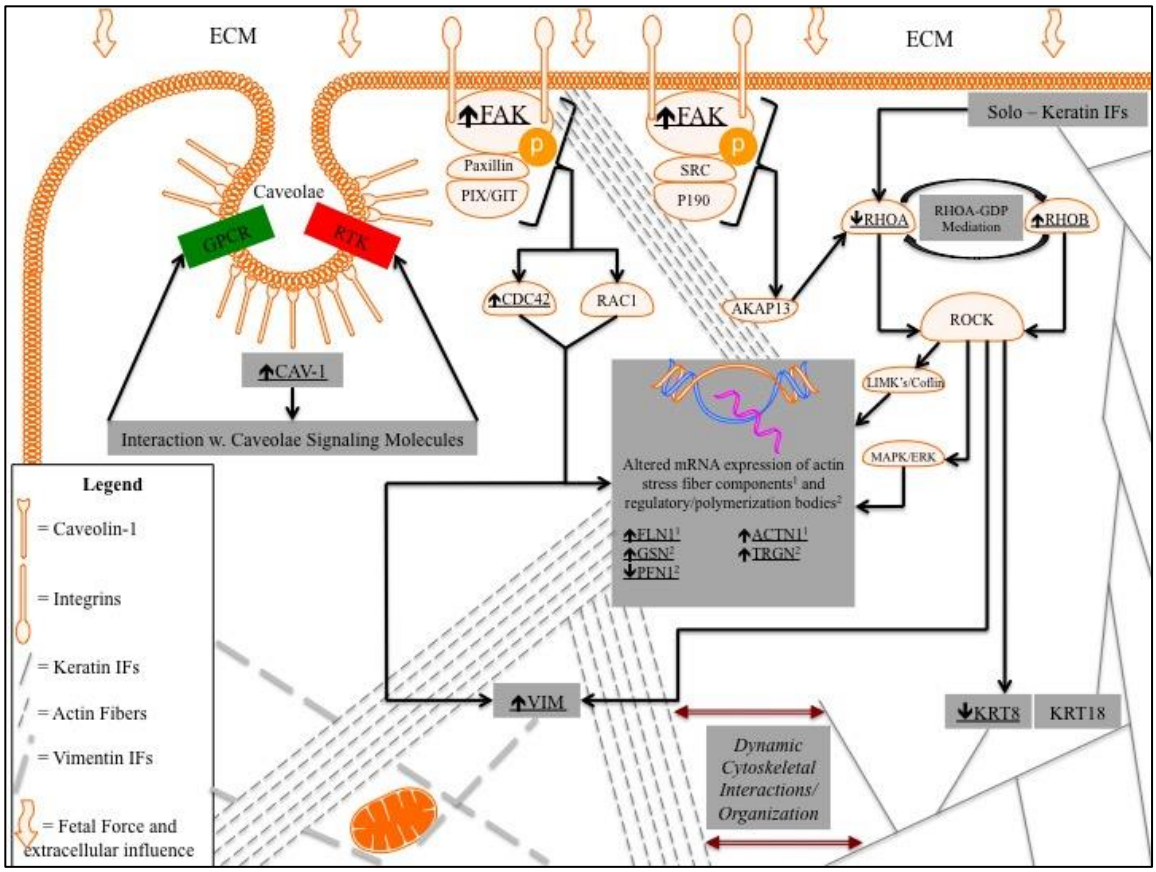

Figure 9

A proposed intracellular mechanism for select cytoskeletal reorganization during cervical remodeling in mice. Extracellular factors inducing this mechanism in remodeling can include both mechanical and hormonal factors. Due to the dynamic cytoskeletal-component expressions and interactions during cervical remodeling, we hypothesize that mechanical force due to fetal growth plays a role larger than what has been previously thought; a mechanism by mechanotransduction. Black arrows signify signaling directionality. Genes/proteins underlined were investigated in this study, and contain an arrow describing expression trend. This model was formulated from the expression data from this study, and in conjunction with existing literature supporting certain interactions (cited in text). GPCR indicates G-Protein Coupled Receptor; RTK, Receptor Tyrosine Kinase; IFs, Intermediate Filaments. Other sources not cited in text, (Chang & Goldman 2004, Zhao *et al.* 2007, Li *et al.* 2004, Bamburg *el at.* 1999, Lappalainen & Drubin 1997, Lawson & Burridge 2014, Li *et al.* 2007).

VITA

Jacob Aaron Gordon was born on February 18, 1996 and is from Pinnacle, North Carolina. He attended public elementary, middle and high schools in Stokes County, North Carolina and graduated from West Stokes High School in June of 2014. That fall he matriculated at Appalachian State University to pursue an undergraduate degree in biology, additionally studying chemistry and music at a minor academic level. While at the university, Mr. Gordon regularly performed with the percussion studio and university ensembles within the Hayes School of Music. Additionally, he served as Founding Member, Judge Advocate, and President of the Beta Chapter of Phi Chi Pre-Medical and Pre-Dental Society at ASU and served as a Health Professions Advising Office Ambassador.

Mr. Gordon will begin a post-baccalaureate research fellowship at the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina on July 23, 2018. He will work in the lab of Robin E. Stanley Ph.D. investigating signal transduction processes in ribosome biogenesis. His parents are Derrick Corby Gordon of Pinnacle, NC and Gina Perkins Gordon of King, NC.